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Catalytic subunits of *Aplysia* neuronal cAMP-dependent protein kinase with two different N termini

(presynaptic facilitation/protein phosphorylation/alternative promoters/alternative RNA splicing)

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ABSTRACT **Previously, two forms of cAMP-dependent protein kinase catalytic subunit generated by mutually exclusive use of two internal exon cassettes (A1 and A2) were demonstrated in *Aplysia* neurons. Here, it is shown that there also exist catalytic subunits with alternative N termini derived from two exons, N1 and N2, expressed in combination with either of the internal cassettes. Processed transcripts including N1 or N2 sequences are of about equal abundance in the nervous system, arise through alternative promoter use, and encode catalytically active polypeptides. The N2 amino acid sequence is 21 residues longer than the N1 sequence and is homologous to the nonmyristoylated N terminus of the *TPK1* gene product, a yeast catalytic subunit homolog. These data support the view that cAMP-dependent protein kinase activity in *Aplysia* neurons is produced by a complex array of regulatory and catalytic subunits that generate multiple holoenzymes with a spectrum of properties.**

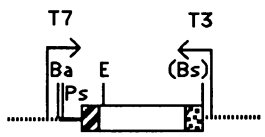
One molecular mechanism for neuronal modulation (1) is through the action of modulatory transmitters that activate signal-transduction pathways in target neurons (2–4). In *Aplysia californica*, cAMP-dependent protein kinase (PKA) plays crucial roles in neuronal modulation at several sites. For example, transmission at sensorimotor synapses is enhanced when modulatory transmitters, which activate adenylate cyclase, are released onto presynaptic sensory cell terminals by facilitatory interneurons. Consequent phosphorylation of a K^+ channel (the S-channel), or an associated polypeptide, by PKA leads to channel closure and prolongation of the action potential (5). The resulting increased influx of Ca^{2+} through voltage-dependent Ca^{2+} channels augments sensory cell transmitter release. Activation of PKA in *Aplysia* sensory neurons also contributes to transmitter mobilization, which is important in facilitation of the depressed synapse (6).

Therefore, to understand the biochemical basis of neuronal modulation in *Aplysia*, the diverse forms of PKA in this species must be defined. To this end, we earlier analyzed cDNAs encoding some of the neuronal regulatory (R) and catalytic (C) subunits of the enzyme (7, 8). In *Aplysia*, the known C subunits of PKA are encoded by a single gene, $C_{APL\text{-}A}$. Processed transcripts contain sequences derived from one or the other of two exon cassettes, A1 and A2, corresponding to exon 6 of the mouse (8). The sequences encoded by the two exons differ at 10 out of 42 amino acids and lie near the midpoint of the polypeptide chain (residues 142–183 out of 352), encompassing residues crucial for enzymatic activity (9). Otherwise identical *Aplysia* C subunits containing A1 or A2 sequences differ in their interactions with both synthetic peptide substrates and a type I R subunit (10).

Recently, C subunits derived through alternative RNA splicing have been described in other species. A catalytic subunit from *Caenorhabditis elegans* has two alternative C termini, one of which is similar to those reported for vertebrates, invertebrates, and yeast. The second C terminus is unrelated to the others throughout its sequence of 56 amino acids (11). A transcript encoding a C subunit with an alternative N terminus, Cβ2, is present in several bovine tissues (12). As demonstrated here, the *Aplysia* C subunits also possess alternative N termini, encoded by two exons (N1 and N2‡; Fig. 1*A*) and expressed in combination with either of the internal cassettes, so that four forms of C subunit are generated. Given that at least five R subunits are present in the *Aplysia* nervous system (13), the present data add to mounting evidence that PKA activity in *Aplysia* neurons arises from a complex array of R and C subunits that generate multiple holoenzyme (R_2C_2) forms with different substrate specificities, regulation, and subcellular localization.

EXPERIMENTAL PROCEDURES

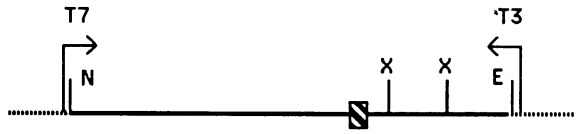
S1 Nuclease Protection Analysis. A cDNA containing the N2 coding sequence and most of the A1 sequence was assembled in the polylinker of the plasmid Bluescript KS(−) and used as a template for the synthesis of a probe to detect alternatively spliced transcripts (Scheme 1 Ba, *Bam*HI; Ps, *Pst* I site in 5′-untranslated region (UTR) of N2 transcript; E, *Eco*RI; Bs, *Bst*EII site in exon A1):



Scheme 1

The plasmid was linearized with *Bam*HI and transcribed with T3 RNA polymerase in the presence of [α-^{32}P]rUTP under conditions that yield a uniformly labeled probe. S1 nuclease protection was done as described (8).

To detect N1 transcription start sites a fragment of genomic DNA containing the coding region of exon N1 and ≈1.5 kilobases (kb) of upstream sequence was isolated from a subgenomic library, subcloned into Bluescript KS(−), mapped, and partly sequenced (Scheme 2 N, *Not* I site in polylinker; X, *Xba* I; E, *Eco*RI):



Scheme 2

Abbreviations: PKA, cAMP-dependent protein kinase; C subunit, catalytic subunit of PKA; R subunit, regulatory subunit of PKA; UTR, untranslated region; nt, nucleotide(s).

‡The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M84335 (N2 sequence) and M84336 (N1 promoter region)].

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The 1700-base-pair (bp) *Not* I–*Xba* I fragment contains 46 bp of coding sequence, downstream from ≈1560 bp of genomic sequence including the 5′ UTR, and upstream from 93 bp of intron. The template for RNA probe synthesis was generated by cutting the plasmid with *Xba* I and *Eco*RI, filling in the ends with Klenow enzyme, and religating. Antisense RNA could then be transcribed from the T3 promoter after linearization with *Not* I. Full-length transcripts were not generated due to abrupt termination of transcription after ≈800 nucleotides (nt). The incomplete transcript was nevertheless satisfactory for the detection of transcription start sites in the N1 promoter region‡ (see Fig. 3*A*).

Antibodies and Immunoselection of N1 and N2 Kinases. Antibodies were raised in rabbits against the N1 peptide (C)KKGDPAENVK (see Fig. 1*B*), the N2 peptide (C)SDQGAKSSDGEG (Fig. 1 *A* and *B*), and a C-terminal peptide common to all the C subunits, (C)NFDDYEEEPLRISSTEK. The peptides were first coupled to carrier bovine serum albumin using sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC: Pierce). Immobilized protein kinase inhibitor peptide was prepared as described (10, 17).

Immunoaffinity columns for selecting C-subunit isoforms containing N1 and N2 sequences were prepared by covalently coupling antipeptide antibodies to protein A-Sepharose with dimethyldipimelidate (18). Anti-bovine serum albumin antibodies were first removed from each antiserum by two passages through Affi-Gel-10 (Bio-Rad) to which bovine serum albumin had been coupled. Immunoselection of N1 and N2 protein kinases was done at 4°C. Five to 10 ovotestes from *Aplysia* (300- to 400-g animals) were homogenized in 40 ml of ice-cold buffer containing 10 mM Tris·HCl, pH 8.2/150 mM NaCl/0.05% Tween 20 (TBS/Tween)/5 mM phenylmethanesulfonyl fluoride/15 mM 2-mercaptoethanol/leupeptin at 10 μg/ml and 0.5% Zwittergent 3-12 (Calbiochem) using a 50-ml ground glass homogenizer. The homogenate was centrifuged at 12,000 × *g* for 30 min. The resulting supernatant was further centrifuged at 100,000 × *g* for 1 hr. The final supernatant was diluted 10-fold in ice-cold TBS/Tween containing 5 mM phenylmethanesulfonyl fluoride and 5 mM 2-mercaptoethanol and loaded onto the immunoaffinity columns, which were linked in tandem, at a rate of 10–12 ml/hr. The columns were individually washed with 50 vol of TBS/Tween, and the kinases were eluted promptly with 100 mM glycine·HCl buffer, pH 3.0. Fractions (0.5 ml) were collected in tubes containing 1 ml of 750 mM 3-(*N*-morpholino)propanesulfonic acid, titrated with NaOH to pH 7.5, 150 mM $MgCl_2$, 1.5% Nonidet, P-40, and 15% glycerol and assayed for kinase activity (10). R subunits were detected with the photoaffinity reagent 8-azido-[^{32}P]cAMP (ICN: 1.4 μM, 71 Ci/mmol; 1 Ci = 37 GBq; ref. 13).

RESULTS

Predicted Amino Acid Sequence of the Alternative N Terminus Encoded by Exon N2. Sequence from a 2.4-kb cDNA, AC–A2, encoding an *Aplysia* C subunit and containing the A2 internal cassette was reported earlier (8). Comparison of AC–A2 with known C subunit sequences, including those encoded by other Aplysia cDNAs, led to the erroneous conclusion that the coding sequence in this clone begins at codon 17. When nuclease protection experiments suggested that previously undetected splice forms of C subunit mRNA were present in *Aplysia* tissues, the origin of the 5′ end of

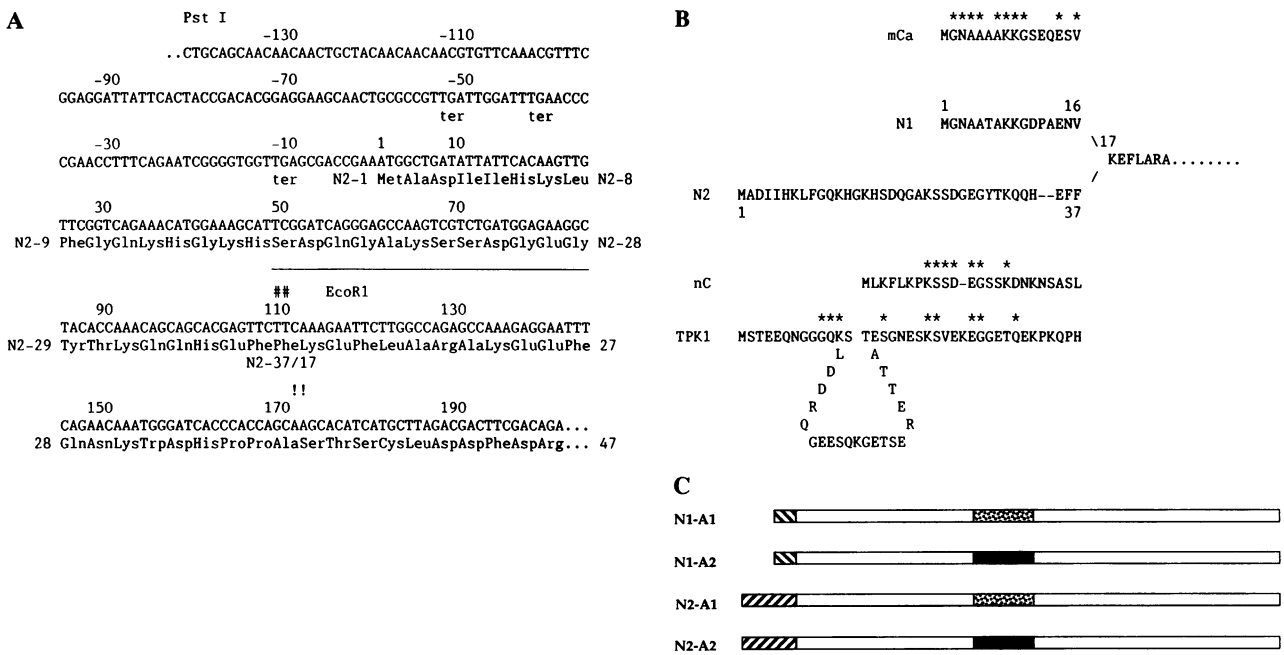

FIG. 1. An alternative N-terminal sequence for the C subunit of *Aplysia* PKA. (*A*) The sequence of an internal region of the cDNA AC–A2 is shown. The initiator methionine is designated residue N2-1, and the first base of the initiation codon is designated nt 1. The ATG is preceded at position −12 by an in-frame termination codon (ter). The splice site discussed in text is between nt 109 and 110 (##). After nt 109 the sequence is identical to that reported (8), in which nt 47 corresponds to nt 110 of this sequence. After the codon at the splice boundary (N2-37) we have reverted to the amino acid numbering scheme used previously. Also shown are the amino acid residues in the synthetic peptide used to produce anti-N2 antibody 152-2 and the *Pst* I site used in constructing a template for cRNA probe synthesis (Fig. 2*A*). The position corresponding to the exon 2/3 boundary of the mouse Cα gene (14) is also indicated (!!); the intervening intron has not been demonstrated in *Aplysia*. (*B*) The alternative N termini are aligned with known C subunit sequences. Identities with N1 or N2 sequences are indicated (*). The N1 sequence reported (8) resembles N termini found in *Drosophila* and mammals, including mouse for which the Cα version is shown (mCa; ref. 15). The N2 sequence can be aligned with the N terminus of *Saccharomyces cerevisiae TPK1* gene product, a structural and functional homolog of C subunits from higher eukaryotes (TPK1; ref. 16), as well as the N terminus of C subunit CeCATα from the nematode *C. elegans* (nC; ref. 11). (*C*) Schematic showing all four possible *Aplysia* C subunits generated through use of internal exon cassettes (8) as well as the alternative N termini (this work). The patterns indicating N1, N2, A1, and A2 sequences are used throughout the paper.

AC–A2 was reexamined, and we now conclude that the cDNA encodes another form of C subunit with an alternative N terminus. The sequence of AC–A2 5′ of codon 17 (Fig. 1*A*) encodes the proposed N terminus (N2), which is 21 amino acids longer than that described (N1). The initiator methionine codon is assumed to be the first in-frame ATG upstream from codon 17. This residue is preceded by an in-frame stop codon (TGA), 12 nt further upstream. The initiator methionine is followed by alanine, and, therefore, this N terminus cannot be myristoylated by the accepted cotranslational pathway (19, 20).

The N1 terminus reported earlier (8) is homologous to the myristoylated N termini found in several mammalian C subunits and the *Drosophila* subunit (e.g., 10/15 identical residues for mouse Cα: Fig. 1*B*). The N2 terminus reported here is homologous to the N terminus of the *Saccharomyces cerevisiae* C subunit homolog encoded by the *TPK1* gene (ref. 16; 9/36 identical residues, 1 gap; Fig. 1*B*). The N termini encoded by the three yeast C subunit genes—*TPK1*, *TPK2*, and *TPK3*—have little sequence in common, and the N2 amino acid sequence is closer to the *TPK1* sequence in this region than the *TPK1* sequence is to the N termini encoded by the *TPK2* and *TPK3* genes. The N terminus reported for the C subunit from *C. elegans*, CeCATα, is also related to the N2 sequence (7/23 identical residues, 1 gap; Fig. 1*B*). Recently, a cDNA (Cβ2) encoding an alternative N terminus for bovine Cβ has been described (12). This sequence bears weak homology to the *TPK* gene products but no direct resemblance to *Aplysia* N2.

Transcripts Containing N1 and N2 Sequences in *Aplysia* Tissues. The combined cDNA cloning data suggest that there are four polypeptide products of the C_{APL-A} gene (Fig. 1*C*). To detect their transcripts, nuclease protection experiments were done by using a probe that included the N2 sequence and most of the A1 sequence (Fig. 2*A*). The sequences encoding the two N termini were about equally prevalent in transcripts in the nervous system (Fig. 2*A*). This was the case whether the N1/N2 ratio was measured using probe fragments protected by transcripts encoding A1 or by transcripts encoding A2. The N1/N2 ratio was also measured in RNA from ovotestis in which a large difference in the relative abundance of A1 and A2 sequences had been observed (8). In this tissue the N1/N2 ratio was again ≈1:1. Again, the value was independent of whether it was measured by using fragments protected by A1 transcripts or by A2 transcripts. This fact suggests that internal cassette splicing in C_{APL-A} gene transcripts is independent of N1 and N2 usage, which is consistent with the evidence that N1 and N2 RNAs arise from the use of alternative promoters (see below), rather than by a splice choice alone. These measurements also served to quantitate A1/A2 usage. Transcripts containing A1 sequences were about three times as abundant as A2 transcripts in the nervous system, whereas transcripts containing A1 were about five times less abundant than A2 transcripts in the ovotestis, representing a ≈15-fold change in relative expression levels between these two tissues (Fig. 2*A*).

N1 and N2 Sequences Are Transcribed from Separate Promoters. The N2 sequence ends precisely at the point corresponding to the boundary separating exons 1 and 2 in the mouse Cα and Cβ genes (ref. 14; Fig. 1*A*). Therefore, the organization of the 5′ end of the C_{APL-A} gene was mapped by Southern blotting using probes specific for the putative *Aplysia* exons N1, N2, and 2 (data not shown). The order of these sequences in the *Aplysia* genome is N2–N1–2. Exon N2 and exon N1 lie within 16 kb of each other, as demonstrated by pulse-field electrophoresis, whereas exon N1 and exon 2 lie within 5.5 kb of each other. Possible transcription start sites in the vicinity of exon N1 were examined in a nuclease protection experiment in which RNAs from ovotestis and nervous system were probed with an antisense transcript of a genomic clone that encompasses exon N1 (Fig. 3*A*). The protected fragments suggest that multiple cap sites exist

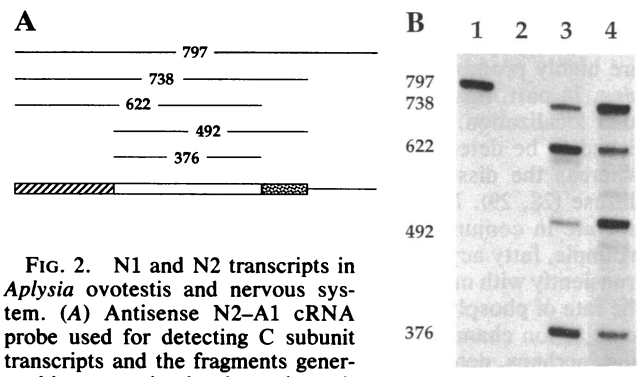

FIG. 2. N1 and N2 transcripts in *Aplysia* ovotestis and nervous system. (*A*) Antisense N2–A1 cRNA probe used for detecting C subunit transcripts and the fragments generated by protection by the various alternative transcripts. Probe, 797; N2–A1, 738; N2–A2, 622; N1–A1, 492; N1–A2, 376. (*B*) S1 nuclease protection of the antisense cRNA probe by C subunit transcripts from the ovotestis and nervous system. Denatured protection products were separated in a 4% polyacrylamide gel containing 7 M urea. Lanes: 1, undigested probe; 2, probe digested in the presence of 25 μg each of carrier yeast tRNA; 3, total ovotestis RNA; and 4, total nervous system RNA.

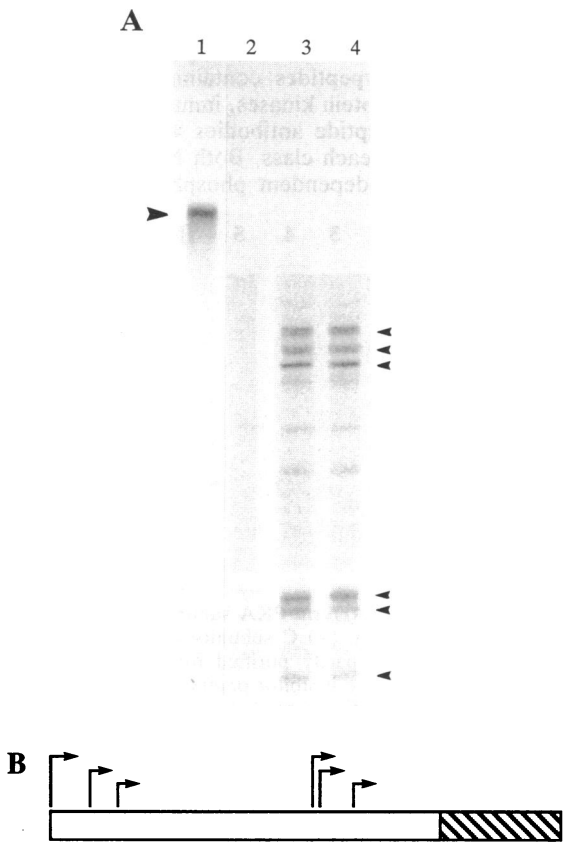

FIG. 3. Detection of a promoter region upstream from the N1 coding sequence by nuclease protection analysis. (*A*) S1 nuclease protection of an antisense cRNA transcribed from a genomic DNA fragment encompassing the N1 coding sequence and upstream genomic sequences by C subunit transcripts from the nervous system and ovotestis. Lanes: 1, undigested probe; 2, probe digested with 25 μg of carrier yeast tRNA; 3, probe digested with 25 μg of total ovotestis RNA; and 4, probe digested with 25 μg of total nervous system RNA. (*B*) Schematic showing the deduced distribution of transcription start sites in the N1 promoter region (open box) in relation to the N1-coding region (hatched). The sites are ≈141, 167, 173, 374, 398, and 435 nt upstream from the initiation codon. A DNA sequencing ladder was used to provide molecular weight markers.

throughout a region ≈140 to ≈435 nt upstream from the initiator methionine codon (Fig. 3B). The promoter regions of the Cα and Cβ genes of the mouse have been analyzed (14). They lack TATA boxes and exhibit multiple transcription initiation sites. However, unlike the N1 promoter region, they have a high G+C content and contain multiple Sp1 core motifs. Because exon N1 is associated with a promoter and lies downstream from exon N2, transcripts including exon N2 must initiate at a separate site.

Immunochemical Analysis of Kinase Fractions Containing N1 and N2 Peptide Sequences. Ovotestis, which is a far more abundant source of protein than the nervous system, was used for the immunochemical analysis of *Aplysia* C subunits. When partially purified subunits were separated by SDS/PAGE and immunoblotted by using an antibody against a C-terminal peptide common to all four forms, a pair of barely separated doublets was detected (Fig. 4A, lane 4). The upper doublet (≈44 kDa) reacted with an antibody prepared against an N2 peptide (Fig. 4A, lane 5), whereas the components of the lower doublet (≈42.5 kDa) reacted with an anti-N1 antibody (data not shown) and comigrated with authentic N1–A1 and N1–A2 polypeptides (Fig. 4A, lanes 1–3). Therefore, it is likely that the components of each doublet are the corresponding A1 and A2 forms (Fig. 1C). The detection of a pair of doublets in which the relative intensities of the high and low molecular mass classes roughly reflect the N1 and N2 message levels suggests that the complexity of the *Aplysia* C subunits may be no greater than that now revealed.

To show that polypeptides containing N1 and N2 sequences are active protein kinases, immunoaffinity columns made with the antipeptide antibodies were used to obtain fractions enriched in each class. Both N1 and N2 fractions catalyzed the cAMP-dependent phosphorylation of kemptide, a synthetic PKA substrate, with a low apparent K_m value of 15 μM (data not shown). When a column prepared using an antibody against the Ser/Thr protein kinase SAK (21) was used in a parallel experiment, no PKA-like activity was eluted, a finding ruling out the possibility of nonspecific adsorption. The R subunit associated with the peak fraction of PKA-like activity from the anti-N2 immunoaffinity column was a 52-kDa polypeptide (Fig. 4B).

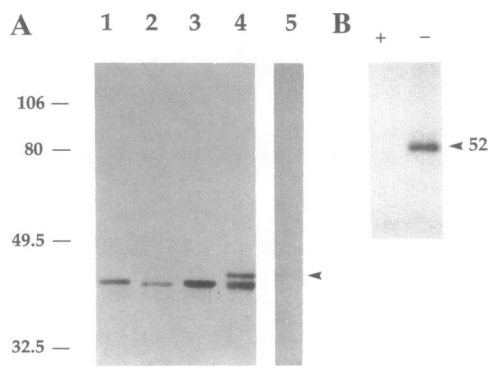

FIG. 4. Detection of *Aplysia* PKA subunits by immunoblotting and photoaffinity labeling. (A) C subunits were overexpressed in insect Sf9 cells (10) or partly purified from ovotestis by using immobilized protein kinase inhibitor peptide (10, 17). The subunits were removed from the affinity matrix by heating in electrophoresis buffer, rather than by elution with arginine. After SDS/PAGE in a 10% gel, the separated polypeptides were electroblotted onto nitrocellulose and probed with anti-C subunit antibodies by a standard protocol using an alkaline phosphatase-conjugated second antibody. Lanes: 1, N1–A1; 2, N1–A2; and 3, a mixture of N1–A1 and N1–A2 subunits from Sf9 cell lysates (10) were visualized with the antibody AC18 against the common C-terminal peptide. Lane 4, kinase subunits of two size classes enriched from ovotestis with immobilized protein kinase inhibitor peptide were also recognized by AC18. The upper doublet was recognized by the anti-N2 antibody 152-2 (lane 5, arrowhead). Prestained markers (Bio-Rad) are as follows: phosphorylase b, 106 kDa; bovine serum albumin, 80 kDa; ovalbumin, 49.5 kDa; carbonic anhydrase, 32.5 kDa. (B) Photoaffinity labeling of an R subunit associated with the peak fraction of PKA-like activity from the anti-N2 immunoaffinity column. The fraction was treated with 8-azido-[^{32}P]cAMP and subjected to electrophoresis in a SDS/11% polyacrylamide gel; the resulting autoradiogram is shown. A 52-kDa polypeptide was protected from labeling by cAMP. −, no cAMP; +, 25 μM cAMP.

DISCUSSION

The present analysis of C_{APL-A} transcripts and polypeptides suggests that there are at least four distinct PKA C subunits in *Aplysia*. Either of two different N termini, which result from alternative promoter use associated with two 5′ exons (N1 and N2), are used in combination with either of two internal sequences, which result from alternative RNA splicing of mutually exclusive exon cassettes (A1 and A2). We have previously argued that the internal cassettes are the products of an early gene duplication (8, 10), as they differ as much from each other as they do from the corresponding mammalian exon 6 (14). It seems likely that exons N1 and N2, which are unrelated to each other, arose from the recruitment of two independent DNA segments into the same gene (22).

The high extent of sequence identity between the N1 terminus and the mouse Cα sequence (Fig. 1B) and the fact that they are of the same size suggests that this N terminus has been conserved for a specific function. Given the phylogenetic distance between *S. cerevisiae* and *Aplysia*, the similarity between the N2 and *TPK1* sequences is also striking and suggests that study of the yeast polypeptide may yield clues about the roles of the N2 forms in *Aplysia*. The N terminus of the *C. elegans* C subunit is also homologous to N2 (Fig. 1B). As the intervening sequence between exons 1 and 2 has been retained in the nematode (11), it remains possible that the genome of this organism also encodes an N1-like alternative N terminus.

One notable difference between the N2 polypeptide sequence and the N1 sequence is the lack of a myristoylation signal in the former (19, 20, 23). The *TPK1* and *C. elegans* N termini and Cβ2 also lack the crucial penultimate glycine. Although myristoylation has clear functional consequences in, for example, pp60^{src} (19, 20), the function of myristoylation in PKA C subunits remains unclear (24, 25). The properties of mammalian C subunits in which myristoylation was prevented, including their interactions with R subunits (24, 25) and all biological activities tested (24), were closely similar to those of the myristoylated polypeptides. Despite this fact, myristoylation might still play an important role under normal physiological conditions. Many protein kinases are highly promiscuous *in vitro* but more discriminating *in vivo*. In part, this difference might be explained by subcellular localization. For PKA, holoenzyme localization is believed to be determined by R subunits (e.g., refs. 26, 27), whereas the dissociated C subunit is soluble and free to diffuse (28, 29). More subtle features of localization could operate in conjunction with this broad generalization. For example, fatty acylation might allow C subunits to associate transiently with membranes. This association would increase the rate of phosphorylation of membrane protein substrates, such as ion channels (4), relative to cytoplasmic substrates and, perhaps, determine which residues are phosphorylated within membrane proteins by orienting the active site of the kinase. The nonmyristoylated N2 terminus might function to target a distinct set of substrates for phosphorylation. Although this is speculation, the distance of the N terminus from the active site and its apparent disorder in the crystal structure of the mouse Cα subunit (30) does suggest that its function is not in the modulation of catalysis.

Besides the four C subunits described here, there are at least five R subunits in neurons (13), which are the products of at least two genes. The transcripts of at least one R subunit gene undergo alternative splicing in the coding region (7). Therefore, the physiological state of an *Aplysia* neuron probably depends upon a spectrum of cAMP-dependent protein kinases with overlapping substrate specificities and regulatory properties (10), different subcellular locations (13), and possibly different abilities to translocate between compartments (29), although there is no evidence for the latter as yet.

Aplysia tissues other than the nervous system express complex arrays of PKA subunits. Further, diverse PKA subunits are generated in various ways in the tissues of other species as a result of the continuing duplication and loss of genes and individual exons (e.g., refs. 11, 16, and 31–35). Moreover, protein kinases are not the only components of eukaryotic signal-transduction systems exhibiting heterogeneity (10). Other systems include G proteins (36), membrane channels (37), and transcription factors (38). Therefore, further studies of PKA subunits in *Aplysia* may yield knowledge useful for formulating general principles about the physiological role of protein isoforms, in addition to information required for a full understanding of presynaptic facilitation.

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